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Original Article

Detection and Characterization of *Nosema bombycis* Using TEM and SEM Techniques

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Abstract

Pebrine disease is the most important and dangerous disease of silkworm caused by *Nosema bombycis* as an obligate intracellular parasitic fungus. It has caused tremendous economic losses in the silk industry in recent years. Given the fact that light microscopy method (with low accuracy) is the only method for diagnosing pebrine disease in the country, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) methods were adopted in this study for accurate morphological identification of the spores causing pebrine disease. Infected larvae and mother moth samples were collected from several farms (Parand, Parnian, Shaft, and Iran Silk Research Center in Gilan province, Iran). The spores were then purified using the sucrose gradient method. From each region, 20 and 10 samples were prepared for SEM and TEM analysis, respectively. In addition, an experiment was performed to evaluate the symptoms of pebrine disease by treating fourth instars with the spores purified for the present study, along with a control group. The results of SEM analysis showed that the mean±SD length and width of spores were 1.99±0.25 to 2.81±0.32 µm, respectively. Based on the obtained results, the size of spores was smaller than the *Nosema bombycis* (*N. bombycis*) as the classic species that cause pebrine disease. In addition, transmission electron microscopy (TEM) pictures showed that the grooves of the adult spores were deeper than those of other *Nosema* species, Vairomorpha, and Pleistophora, and resembled *N. bombycis* in other studies. Examination of pathogenicity of the studied spores indicated that the disease symptoms in controlled conditions were similar to those in the sampled farms. The most important symptom in fourth and fifth instrars were the small size and no growth in the treatment group compared with the control group. Findings of SEM and TEM analysis showed better morphological and structural details of parasite compared with light microscopy, and demonstrated that the studied species were a native strain of *N. bombycis* specific to Iran, whose size and other characteristics were unique and introduced for the first time in this study.

Keywords: *Nosema bombycis*, Pebrine disease, SEM, Silkworm, TEM

1. Introduction

Pebrine disease as the most important and dangerous disease of silkworm, has caused tremendous economic losses in the silk industry. Considering the fact that pebrine disease spreads horizontally and vertically by transmission from spores, it is too difficult to eradicate it from sericulture and silk industries, and it causes significant loss of sericultural products every year (1). Moreover, the most applied method of diagnosis for pebrine disease in Iran is the light microscope method, which does not have acceptable accuracy (2).

Sericulture is considered to be a sustainable economic activity on the farm, which is beneficial for the poor rural people in countries such as Iran. Due to the demographic structure of the country, a large population of young job seekers, and insufficient

income from major agricultural occupations to meet the employment and income needs of villagers, other activities such as silkworm breeding can be helpful. In Iran, the main research centers for the management of sericulture industry have been established in Gilan province, as the main hub of the sericulture with a long history of sericulture, efficient manpower, and appropriate climatic conditions. Currently, sericulture has an important role in producing cocoons, silk threads, and propagation of mulberry seedlings in the country (2).

In recent years, pebrine disease has been the main problem in the silkworm fields in Iran, and even affected the commercial lines of silk thread production. During 2016-2019, the spread of pebrine disease on a large scale in an egg production farm in Gilan province, Iran, practically disrupted the production of commercial hybrid eggs and forced the import of silk products from 1996 until now. This industry is very important for the country since Iran is one of the few countries in the world with a complete line of silk cocoon production (including line, ancestors, parent, and commercial hybrids) from 20 years ago. Indeed, this industry has been completely endogenous and combating this disease is one of the main priorities of the breeding industry in Iran (3).

Microsporidia, as obligate intracellular eukaryotic parasites, attack a variety of hosts, including primates and mammals (4). They produce spores consisting of a polar tube at the end of the spore, which under suitable conditions, penetrate a new host cell through the thin anterior end of the spore, drain it, and infect the host cells. Microsporidia also invade the host cells through phagocytosis and a special mechanism is used after phagocytosis to infect the cytoplasm of cells and escape adult phagosomes (5). They belong to the branch of microspores and have separate nuclei that are located inside the nuclear membrane (4).

Main pebrine symptoms in infected larvae include dark spots or swollen areas of the cuticle compared to healthy larvae. Usually the most infected body tissues in insects include adipose tissue, and intestinal epithelium are observed in white porcelain. The infected pupae have a soft abdomen with mild swelling, as well as black spots on the abdomen. Heavily infected pupae cannot become moths. In infected mother moth, the wings and antenna are damaged, and scales and baldness are observed in the abdomen and wings. The infected gonads present several drawbacks, such as incomplete mating, low fertility, and spawning with a high percentage of infertile and dead eggs.

Scanning electron microscopy (SEM) produces a good image of surfaces with magnifications up to 500,000 times and a high resolution of less than 1 to 20 nanometers, which is suitable for the proper study of chemical structures. In addition, the application of these tools is important for the study of the cellular activity and structure of proteins in the intracellular growth stage of microsporidia. Moreover, TEM has always been a useful tool for identification of the early stages of infection development and data collection on cell structure.

Understanding the characteristics of spores, such as size, spore wall, and other components of spores affected by pebrine disease is very important for understanding the interaction between parasite and host. Wang, Chambon (6) reported that adult spores of *N. bombycis* were oval and the length and the width of the spores ranged from 2.2 to 3.1 μm and from 1.9 to 2.6 μm, respectively. Adult spores had a double layered wall, a sproplasma and two nuclei attached to each other. Cytoplasmic organs included the invasive apparatus (polar tube complex, anchor disc, and polar filament), the polaroplast, the posterior vacuole at the end of the spore, the ribosome, the endoplasmic reticulum, and the golgi apparatus. The spores were very transparent and bluish white under a light microscope (6).

There are various diseases, which infect silkworms including pebrine (Microsporidia: Nosematidae), flacherie (bacteria), grasserie (virus), and muscardine (fungus). Several reports of crop loss are available regarding the specific diseases in silkworm in India, which vary from 20 to 40% (7). Nataraju, Stahyaprasad

(8) reported that Nosema infection with occasional crop failure accounted for about 36% of crop loss.

It should be noted that the routine method of diagnosing the causative agent of pebrine disease in commercial sericulture farms is out of date and inefficient, and the species or different pathogenic and non-pathogenic microsporidia in silkworm are still unknown in Iran. Identification of the pathogen genus and species can be the basis of the future phylogenetic studies. The growth stages of parasites can be observed by TEM or SEM, especially in the cell proliferation stage, which is invisible by light microscopy. Therefore, the present study aimed to identify and differentiate the types of spores that cause pebrine disease in several regions of north Iran, to further control and eradicate this deadly disease in the silkworm industry.

2. Materials and Methods

2.1. Isolation and Purification of Microsporidia Spores

The pure microsporidia spores were extracted from infected moths by homogenizing 20-30 infected moths (crushed together in 25 mL 0.5% potassium carbonate solution) in a process described by Sato, Kobayashi (9). The suspension was then filtered twice through absorbent cotton, washed 2-3 times in distilled water, and centrifuged at 5000 g for 10 min. Afterward, the sediments were treated with 5 mL potassium hydroxide solution (2%). The spore solution was then added to a discontinuous sucrose gradient (25%, 50%, 75%, and 100% v/v, each at 5 mL volume in a 50-mL falcon) and centrifuged at 20,000 g for 40 min twice. Eventually, pure spores were placed between 75% and 100% sucrose levels, were obtained and suspended in distilled water and counted using hemocytometer under a phase-contrast light microscope.

2.2. Scanning Electron Microscopy (SEM)

For each studied farm, 20 samples of purified spores were extracted from infected mother moth. A homocytometer was used to count the spores and appropriate dilutions were then prepared on metal paper according to the standard protocol. Subsequently, initial stabilization of purified spores was conducted using glutraldehyde (2.5%) and phosphate buffer. The pellets were dehydrated through different grades of ethanol. One drop of each of the dehydrated samples was placed on the upper surface of the foil, dried, and fixed using osmium tetraoxide (2%). Eventually, the sample was held between the coating and the metallic copper stud using a silver-based paint. The copper stud was then placed in a vacuum evaporator that was connected to a high electrical voltage (20 kV) to place the gold vapor coating on the copper stud sample at 300°C. Imaging was performed using the FeSEM field emission microscope (MIRA3TESCAN-XMU, Razi Applied Sciences Foundation, Tehran, Iran) and all samples were observed.

2.3. Transmission Electron Microscopy (TEM)

Pure spores (2.5 mL) containing one million spores were prepared. The initial preparation was performed at Biochemistry Department of the University of Tehran as follows: the samples were mixed with glutaraldehyde (2.5%) for initial stabilization and the block was prepared following the standard protocol after fixation with osmium tetroxide (2%) in molten agar (1.5%) (10, 11). Afterward, only the golden and gray parts were stained with uranyl acetate and lead citrate and uranyl acetate were used as the block stain. Subsequently, 100 to 400 nm cuts of the samples were performed using a diamond knife. Imaging was performed using an electron microscope (model ZAISEM900) (50 kV) with a magnification of 20,000-30,000 at the Pasteur Institute in Tehran, Iran.

2.4. Evaluation of Pathogenicity and Disease Symptoms

To evaluate the pathogenicity and the symptoms of the disease in controlled conditions, an experimental design for breeding silkworm larva was performed at Iran Silk Research Center (ISRC). Healthy larvae were placed in optimal condition at the beginning of the fourth instar and were treated with the studied spores

with two concentrations of 1×10^5 and 1×10^6 spores per mL (by spraying on mulberry leaves), which were isolated and counted at Genomics laboratory (Razi vaccine and serum research institute, Karaj, Iran). The control group consisted of healthy larvae, which were fed by uninfected mulberry leaves. The larvae were observed until the end of the experimental period (after the pupal stage). At the end of the rearing period, SEM imaging was carried out for spores isolated from treatment group and compared with spores isolated from studied farms.

3. Results

Results of the isolation and purification of the spores under light microscope are presented in figure 1. The spores were very clear and bluish white and had brown motion under the microscope.

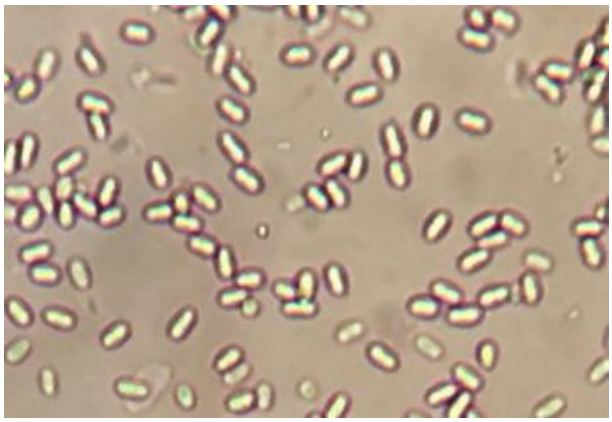

Figure 1. Image of spores under 10X magnification light microscope

The results of scanning electron microscopy are presented in figures 2 and 3. Spore images were recorded using an electron microscope with magnifications of 20000 X and 30000 X. The mean±SD length and width of the spores were 1.99±0.25 and 2.81±0.32 µm, respectively. As can be observed, the groove depth of adult spores was relatively higher compared to other genera, such as Vairomorpha and Pleistophora. Also, the groove depth of these samples was higher than other *Nosema* species.

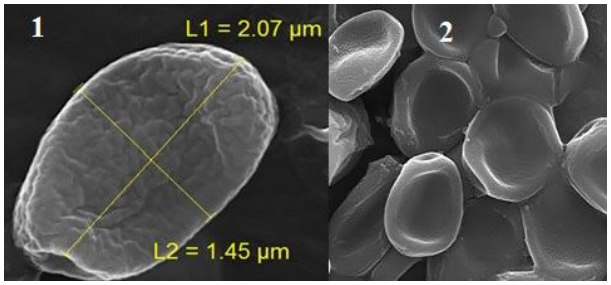

Figure 2. Image of spores using SEM (30000 X (1) and 20000 X (2) magnifications). The indentation of the spores is clearly shown

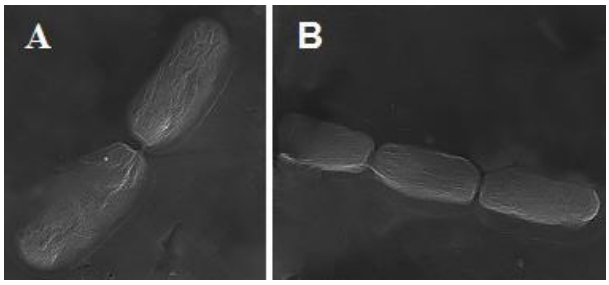

Figure 3. Image of spores with two (A) and three (B) divisions.

Regarding that the most immature spores are removed during centrifugation at very high speeds to purify the spores, a small number of retrieved spores show early stages of growth; however, these sporoblasts were distinguishable from adult spores in some samples. Figures 4 and 5 show secondary sproblasts as well as spores, which are ready to become mature spores. Also, figures 6, 7 and 8 show adult spores of N. bombycis with cross sections.

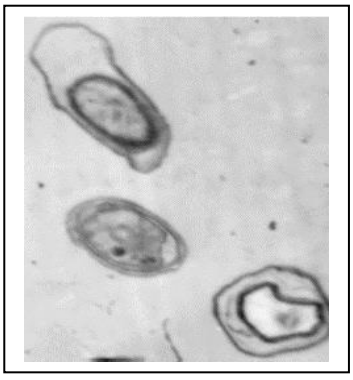

Figure 4. Transparent primary sporoblasts with an uneven electron dense membrane.

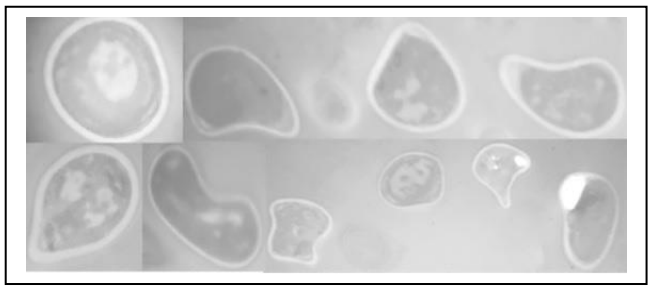

Figure 5. Secondary sporoblasts (thin-walled) and sporonts (thick-walled) at different stages of growth to become mature spores

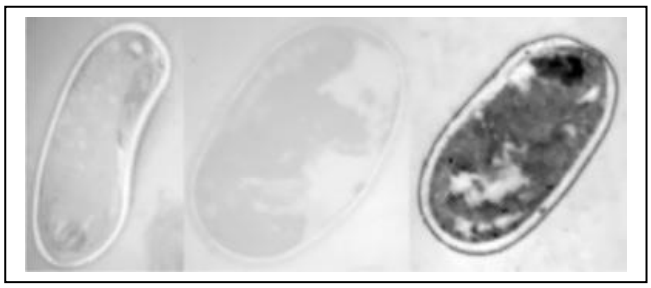

Figure 6. Adult spores of *N. bombycis* with cross sections

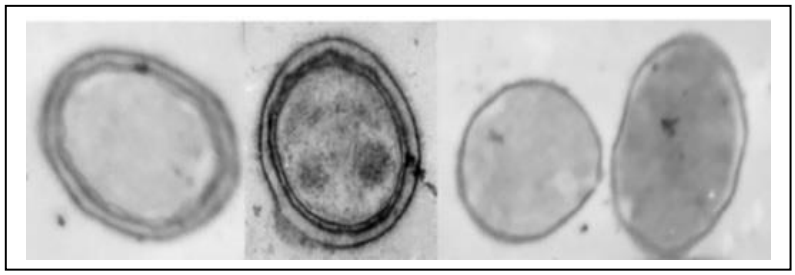

Figure 7. Adult spores of *N. bombycis* after germination whose sporoblast contents have been emptied

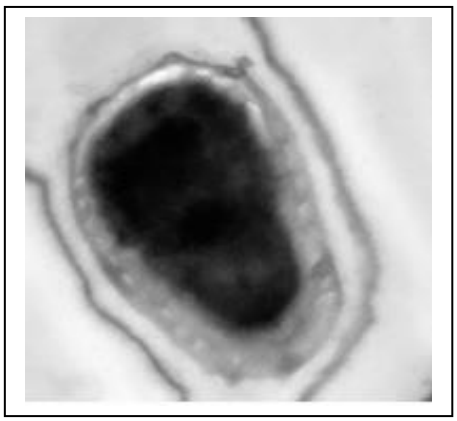

Figure 8. Adult spores of *N. bombycis*: the coils can be seen with medium resolution, but their exact number is not measurable

The pathogenicity and disease symptoms in controlled conditions for the studied spores were the same as the sampled farms. The most important symptom in fourth and fifth instar after infection with *N. bombycis* spores was incomplete growth of larvae in the treatment group compared to control group (Figure 9). Since, the larvae were infected at the beginning of the fourth instar, most of them had the pupal stage, but, the pupal weight, pupal cortex weight and cocoon weight were significantly lower than those of the control group. If the larvae were infected at younger stages, they would not be able to pass the pupal stage and then, the cocoon would not form. Comparison of SEM results of spores isolated from treatment group and spores isolated from studied farms showed both samples prototypes were the same.

Figure 9. Sample of fifth instar larvae before pupal stage. **A**: the larvae of the treatment group (fed with infected leaves) **B**: the larvae of the control group (fed with uninfected leaves)

4. Discussion

In recent years, the causative agent of pebrine in silkworm has become a major problem in the production of sericulture products. In the northern provinces of Iran, its large-scale spread in egg-producing farms has virtually disrupted the production and import of commercial hybrid eggs. Given that, Iran is one of the few countries in the world that has a complete production line of silk cocoons (including line, ancestors, parent and commercial hybrids) for 25 years, and with the efforts of the country's researchers, this industry has become completely endogenous. Therefore, the combat against this disease has been one of the main priorities of the country's breeding industry. Thus, combating pebrine disease in silkworm should be based on prevention; however, no effective drug has been introduced to treat the disease.

Images of spores under SEM (20000 X and 30000 X magnifications) showed that the length and width of spores under electron microscope (mean size of spores plus standard deviation) were between 1.99 ± 0.25 and 2.81 ± 0.32 µm. According to a previous report (3.8 × 2.2 (13)), it is obvious that the size of spores in the present study was slightly smaller than the classic *N. bombycis* spores.

In this study, SEM showed acceptable morphological and structural details of microsporidia spores. For example, the surface of the spores had deep grooves. Chakrabarty, Saha (12), showed that adult spores observed by SEM (35000 X magnification) had slight depressions at both ends along with smooth exospores and hard walls. Comparing the differences between the two *Nosema* species, indicated that the exospores of the spores of *N. ceranae* have a deeper depression than those of *N. apis* (12). Ptaszyńska, Borsuk (13) showed the microsporidia spore's wall was composed of two layers: exosporium and endosporium (Nosema exosporium) and has three layers and also, has an electron-dense glycoprotein structure (14, 15). TEM studies have shown that the internal structure of *N. apis* and *N. ceranae* spores can be distinguished from each other based on the number of polar filaments, for example, *N. ceranae* has fewer polar filaments than *N. apis* (15-17), which was consistent with the results of other studies(12, 13, 18).

TEM can show accurate and high quality structures of sample and also, is a special tool for analyzing a relatively small area of the selected samples, while SEM has proven to be a good tool for viewing details of morphological structures and therefore, can be used to differentiate species. In this study, the coil angle was consistent with the reported results (12) in the case of *N. bombycis*.

Spore wall increases the resistance of spores against environmental effects. Increasing the hydrostatic pressure causes the destruction of the spore wall and then, growth begins. Along with the growth of the polar tube, structural changes occur in the spore (18). These findings were consistent with all the structural features of Nosema spores that have been documented (9, 12).

The results of pathogenesis and disease symptoms indicated that the symptoms of the disease in controlled conditions were consistent with the symptoms in the studied farms. The most important symptom in the fourth and fifth instar larvae was small size and lack of proper growth of larvae compared to the control group. According to the results of other researchers, the spores studied in this study were smaller than the spores of *N. bombycis* as a classic species causing pebrine disease.

In general, it can be concluded that the studied species is a native species of *N. bombycis* and specific to Iran and its size and other characteristics were unique and was introduced for the first time in this study.

Authors' Contribution

Study concept and design: M. M., M. B. and Sh. N.
Acquisition of data: M. B., Sh. N. and M. M.
Analysis and interpretation of data: M. B. and Sh. N.
Drafting of the manuscript: M. B.
Critical revision of the manuscript for important intellectual content: M. B.
Statistical analysis: M. B. and Sh. N.
Administrative, technical, and material support: M. B., Sh. N. and M. M.

Ethics

The procedures were approved by the ethics committee of the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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